ENZYMATIC DECONTAMINATION OF C/B THREAT MATERIALS: FROM CONCEPT TO COMMERCIALIZATION

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ABSTRACT

The ability of enzymes to hydrolyze and detoxify organophosphorus compounds goes back nearly 60 years to when Mazur published work with mammalian tissues conducted during World War II (Mazur, 1946). He determined that enzymatic activity in a variety of tissues could catalytically detoxify diisopropylfluorophosphate (DFP). More recently, a variety of enzymes with activity against G- and V-type nerve agents as well as organophosphorus pesticides have been identified from numerous sources, purified and characterized. A review of these enzymes can be found in a recent review (DeFrank and White, 2002). With several of these enzymes now available in industrial quantities, the concept of a limited capability enzyme-based decontaminant has reached fruition.

1. INTRODUCTION

As stated in the Joint Science and Technology Chemical and Biological Decontamination Master Plan, "decontamination is defined as the process of removing or neutralizing a surface hazard resulting from a CB agent attack. Its purpose is to quickly restore battlefield operational tempo and logistics after a CB attack has occurred. CB agents require cumbersome protective measures that cause significant degradation of combat performance. Thus, decontamination capabilities are required to sustain operations in a CB contaminated environment, to ensure power projection capabilities, to clean up personnel and large areas for retrograde and resupply operations, and to reconstitute individual equipment, vehicles, and weapon platforms. The objective of decontamination technology advancement efforts is to develop systems that are rapid and effective in detoxifying CB agents, environmentally safe, do not impact the operation effectiveness of the equipment being decontaminated, and minimize the logistical impact on operations." In addition to the traditional concept of decontamination of vehicles and equipment, it has been recognized that it is now crucial to be able to decontaminate large areas such as logistics bases, airfields, ports, key command and control centers and other fixed facilities. With the rising threat of terrorist attacks with CB agents or toxic industrial chemicals, large civilian facilities may also require decontamination.

Fig. 1. Military/civilian decontamination operations

Current chemistry-based decontaminants are caustic or have the potential for causing materiel and environmental damage. This, and the fact that some are also flammable, has made them inappropriate for shipboard use or on new, high performance aircraft. In addition, most are bulk liquids that require significant logistics and storage capabilities. While some of these decontaminants are non-aqueous, they still require considerable amounts

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of water for pre- and post-application rinsing (to prevent corrosion of equipment). It would seem very unlikely that they could be used in dealing with large outdoor fixed sites that have been contaminated.

2. ENZYME-BASED DECONTAMINANTS

An appropriate mixture of enzymes and other natural products offers considerable advantages over other decontaminants. Being catalytic, the enzymes are highly efficient and can detoxify many times their own weight of agent in seconds or minutes. Unlike most chemical catalysts, enzymes of many different types and specificities can be mixed together in a single formulation. Since enzymes function best at pH values near neutrality, there are few, if any, compatibility or corrosion concerns as long as the material being decontaminated can tolerate water. As a water-based system, there will not be any flammability concerns. Similar to enzymes in commercial laundry detergents, an enzyme-based decontaminant, being biodegradable, will pose little or no health or environmental danger and leave no hazardous products that would need to be dealt with. Potentially, an enzyme-based decontaminant will be benign enough to be used directly on the skin of personnel and casualties. Another major advantage is that an enzyme-based decontaminant would be provided as a dry granulated powder that is added to whatever water-based spray or foam system the user has available. This provides a significant reduction in the logistical burden as well as making use of existing equipment – both military and civilian.

Enzyme-based decontaminants have been under development at the Edgewood Chemical Biological Center (ECBC) for many years. In the area of nerve agents and related compounds, two bacterial enzymes have been the primary targets of research and development in recent years. These are:

- Organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp. JD6.5
- Organophosphorus hydrolase (OPH) from *Flavobacterium* sp.

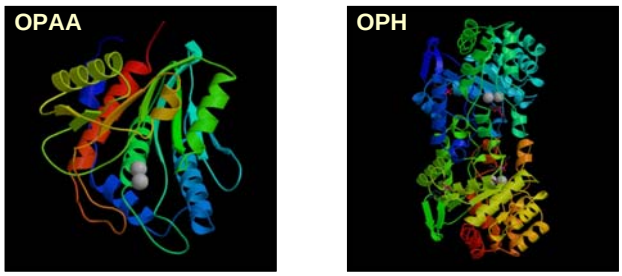

Fig. 2: Structures of OPAA and OPH enzymes

2.1 Organophosphorus Acid Anhydrolase

Isolate JD6.5 was obtained from Grantsville Warm Springs, Utah, which has a relatively constant temperature of 24-32°C and 25,000 ppm dissolved solids (96% NaCl). The isolate is a Gram-negative, aerobic short rod, and an obligate, moderate halophile. It required at least 2% NaCl for growth, with an optimum between 5-10% NaCl. Fatty acid analysis of the isolate identified it as a strain of *Alteromonas*, a common genus of marine bacteria. An intracellular OPAA enzyme from strain JD6.5 was purified and characterized (DeFrank and Cheng, 1991). It is a single polypeptide with molecular weight of 58 kDa. It has a pH optimum of 8.5 and temperature optimum of 50°C. Its catalytic activity to nerve agents and related compounds is shown in Table 1.

Table 1. *A.* sp. JD6.5 OPAA. Substrate Specificity

Substrate*	k_{cat} (sec^{-1})
GD (soman)	3145
DFP	1820
GF	1654
GB (sarin)	611
Paraoxon	124
GA (tabun)	85
VX	0

* The activity on the substrates with fluoride leaving groups was measured with a fluoride ion-selective electrode method. Activity on paraoxon was determined by measurement of the increase in absorbance at 405 nm representing the release of the p-nitrophenol group. For GA (tabun), cleavage of the P-CN bond was determined by P31-NMR.

A number of other *Alteromonas* strains were obtained from the laboratory of Dr. Rita Colwell, University of Maryland, and the American Type Culture Collection (ATCC). These strains were evaluated for enzyme activity against DFP the nerve agents (DeFrank et al., 1993). Several showed high levels of activity and two of the enzymes were purified and characterized (Cheng et al., 1993; Cheng et al., 1997). There are significant similarities as well as differences with these enzymes. In order to produce the enzymes in larger quantities, the genes for *A.* sp. JD6.5 and *A. haloplanktis* were cloned into *E. coli* and expressed. In addition, the gene sequences were determined and translated into an amino acid sequence. The 10-kDa molecular weight difference between the *A.* sp. JD6.5 and *A. haloplanktis* OPAA's was found to be due to the presence of an extended C-terminal region in the JD6.5 enzyme. The two enzymes have a 77% amino acid homology. If the extended C-terminus of the JD6.5 enzyme is excluded, the homology increases to ~90%.

Previously, it was assumed that the natural function of OPAA's would have something to do with phosphorus metabolism (phospholipase, phosphodiesterase, etc.). Therefore, it was a considerable surprise when the results of screening the amino acid sequence of *A.* sp. JD6.5 against the NCBI protein database revealed a high degree of homology (48%) to the *E. coli* X-Pro dipeptidase. Two other matches were for *E. coli* aminopeptidase P (31% homology) and *Lactobacillus sake* dipeptidase (19% homology). X-Pro dipeptidases, also known as prolidases (EC 3.4.13.9), are a ubiquitous class of enzymes that hydrolyze dipeptides with a prolyl residue at the carboxyl-terminal position. They are usually activated by Mn^{2+}, are possibly thiol dependent, and usually do not act on tri- or tetrapeptides or dipeptides with proline at the N-terminus. They generally have a molecular weight of 40-50 kDa, a temperature optimum between 40 and 55°C, and a pH optimum between 6.5 and 8.0. All these properties are very similar to those of the. The identity of the *Alteromonas* OPAA's as prolidases was confirmed through examination of their activity on a variety of dipeptides.

Hoskin and Walker (1998) examined several nerve agent/DFP-hydrolyzing enzymes to determine whether any of these might also be prolidases. Rather than measuring dipeptide hydrolysis directly, they reasoned that if a DFP-hydrolyzing enzyme also hydrolyzes Leu-Pro, then Leu-Pro should inhibit the hydrolysis of DFP. They demonstrated that the hog kidney and the *E. coli* OPAA's most likely are prolidases and that the squid-type OPAA and the *P. diminuta* OPH are not. This does not eliminate the possibility that the squid-type enzyme may be a peptidase with different substrate specificity. However, for both OPH and the squid DFPase, the fact that they have no sequence homology suggests that they have very different natural functions than the prolidases.

The question naturally comes up as to why prolidases are such efficient catalysts for the hydrolysis of organophosphorus compounds, in particular, for the nerve agents. Molecular modeling studies comparing the structures of soman and Leu-Pro have been carried out. It was determined that the three-dimensional structure and the electrostatic density maps of the materials look nearly identical. The organophosphorus compounds such as soman appear to fit into the active site of the enzyme in an orientation that allows the hydrolysis of the target P–F, P–CN, or P–O bond. Using the crystal structure of the *E. coli* methionine aminopeptidase, a postulated model of the A. sp. JD6.5 active site has been developed. It indicates two hydrophobic pockets, a large one where the side chain of the leucine (of Leu-Pro) can fit and a smaller one where the proline ring fits. The amide bond is positioned for a backside attack just above the two metal ions that have a bridging oxygen or hydroxyl group. When soman is substituted for Leu-Pro, the pinacolyl group fits into the large hydrophobic pocket and the methyl group into the small pocket. The phosphorus atom is located in the same position as the amide bond of Leu-Pro and the fluorine leaving-group extends out of the active site. The model suggests that the activity of the dipeptidases on soman and related com-pounds is primarily a matter of serendipity. They mimic the structure of the natural substrates for the enzyme so well that the enzymes are able to efficiently catalyze their hydrolysis. Confirmation of this proposition will be based on the crystal structure of the A. sp. JD6.5 enzyme. While Dr. Quoicho, Baylor University, has determined the structure of the enzyme it has not yet been published.

The *A.* sp. JD6.5 prolidase has proven to be quite amenable to production by recombinant DNA technology. In initial studies, the *opa*A gene encoding the prolidase was cloned into pBluescript SK+ (pTC6513) and expressed in *E. coli*. The expressed enzyme constituted about 5% of the total cell protein. To further enhance production, the gene was cloned downstream of a strong *trc* promoter in a high-level, regulated expression vector, pSE420 (Invitrogen, San Diego, California). After induction with IPTG, the enzyme was produced at levels up of to 50-60% of total cell protein for a yield in shake-flask cultures of 150-200 mg/L. Fermentation studies with 10-liter fed-batch systems have pushed the levels to nearly 1 g/L. Two U.S. patents have been awarded on the sequence and use of this enzyme (Cheng and DeFrank, 1999, 2000).

2.2 Organophosphorus Hydrolase

The Organophosphorus Hydrolase (OPH) (parathion hydrolase, or phosphotriesterase) from *P. diminuta* or *Flavobacterium* has been one of the most studied enzymes in regard to its activity on nerve agents and pesticides. While many researchers have studied OPH, the primary information on the structure and function of the enzyme has come from the laboratories of Raushel and Wild, both at Texas A&M University. Most early work with the enzyme used the constitutively expressed form which is membrane associated (Brown, 1980). When the gene for the enzyme was cloned into other hosts, the membrane association remained, making purification difficult. It was discovered that the enzyme was synthesized as a 365 amino acid precursor from which 29 amino acids were removed to generate the mature protein. When this 29 amino acid leader sequence was removed from the clone, the recombinant enzyme was found as a soluble mature enzyme that maintained activity. The enzyme has been expressed in a variety of hosts including insects, insect cells, fungi, and *Streptomyces.*

The mature enzyme is a ~36-kDa metalloprotein with two Zn^{2+} ions present in the native enzyme. However, a variety of other divalent metal ions (Mn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+}) can be substituted (Omburo et al., 1992). The Co^{2+}

enzyme has the greatest activity on substrates with P-F and P-S bonds. Originally thought to function as a monomeric enzyme, more recent information based on the crystal structure of the protein indicates that the active form is actually a homodimer (Benning et al., 1994).

OPH has a broad pH profile with optimum activity between pH 8-10 and a temperature optimum of ~50ºC. OPH has the ability to hydrolyze a wide variety of organophosphorus pesticides as well as other compounds having P–O, P–F, and P–S bonds. Several selected substrates and the kinetic constants for OPH (Co^{2+} form) are shown in Table 2.

Table 2. Hydrolytic Constants for OPH.

Substrate	Bond type	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} sec^{-1})
Paraoxon	P–O	3170	0.058	5.5×10^7
Parathion	P–O	630	0.24	2.6×10^6
DFP	P–F	465	0.048	9.7×10^6
Sarin	P–F	56	0.7	8.0×10^4
Soman	P–F	5	0.5	1.0×10^4
Demeton-S	P–S	1.25	0.78	1.6×10^3
VX	P–S	0.3	0.44	45
Acephate	P–S	2.8	160	18

Assays conducted at pH 7.2 and 37ºC

As mentioned earlier, recombinant OPH has been produced in a variety of host organisms. However, in most cases the level of production has been relatively low 10-25 mg/L. In some instances, it is believed that the enzyme forms insoluble and inactive inclusion bodies, but this has not yet been confirmed. A variety of techniques such as varying growth temperature, differing the carbon sources in microbial fermentations, fed-batch fermentations, and others are being pursued. The use of a host that will secrete the active enzyme may also solve this problem, although production by *Streptomyces* was still quite low. As reported in a recent patent, slight modification of the recombinant gene has improved the yield considerably. In addition, an improved method of purification has resulted in a near one-step process (Rastogi et al., 2002).

2.3 Formulation

Nerve agent-degrading enzymes are capable of functioning in a variety of water-based systems such as fire-fighting foams and sprays, aqueous degreasers, aircraft deicers, and commercial laundry detergents (Cheng et al., 1998). Examples of this are shown in Table 3.

Preliminary testing of single enzyme systems (OPAA) in a variety of fire-fighting foams and sprays has been conducted under the auspices of NATO Project Group 31 (PG/31), "Non-Corrosive, Biotechnology-Based Decontaminants for CBW Agents." They have consistently resulted in >99.5% destruction of soman (GD) and thickened GD in 15 to 30 minutes.

Table 3: OPAA Activity on DFP in Possible Vehicles

Vehicle	Property	Conc. Used (%)	Relative Activity
Control (ammonium carbonate buffer, pH 8.7)	—	—	100
AFC-380 (Sandia National Lab: Albuquerque, NM	Blast containment foam	6	54
BioSolve® (Westford: Westford, MA)	Fire-fighting wetting agent	6	53
Fire Choke® (Fire Response: Houston, TX)	Class A fire-fighting foam	0.5	100
BV 406LF (FireFreeze: Rockaway, NJ)	Degreaser/ cleaner	10	73
Cold Fire® (FireFreeze: Rockaway, NJ)	Fire-suppressing agent	10	120
Odor Seal® (FireFreeze: Rockaway, NJ)	Odor removing wetting agent	10	102
Tide® Free (Proctor & Gamble)	Laundry detergent	0.05	108
CORNsolv (SOYsolv: Tiffin, OH)	Biodegradable solvent	1	121

Similar to commercial laundry detergents containing enzymes, an enzyme-based decontaminant will pose little or no health or environmental danger and leave no hazardous products requiring cleanup. Another major advantage is that an enzyme-based decontaminant would be provided as a dry powder that can be added to whatever water-based spray or foam systems are available to the user. This provides a significant reduction in the logistical burden (25-50 fold) as well as making use of existing equipment – both military and civilian. For example, to provide two million gallons of decontaminant for a major military engagement would require 11,000 tons of DS2. On the other hand, the equivalent amount of dry, enzyme-based decontaminant would weigh ~56 tons and have no special storage or transportation requirements. This reduction in logistics burden is especially important for ships at sea. For example, when added to an aircraft carrier's Countermeasure Washdown System, less than 500 pounds of enzyme-based decontaminant would be required to treat the entire flight deck (1092 x 257 ft, ~200,000 ft^2, 4.5 acres) with 3" of foam.

2.4 Large-Scale Production

In order for any decontaminant to reach commercialization or fielding, large-scale production of the components needs to be achieved. In early 2004 a Patent License Agreement (PTA) was signed between ECBC

and Genencor International in regards to large-scale production of the OPAA and OPH enzymes. Genencor is the largest U.S. producer of industrial enzymes and second largest in the world. Initial efforts will use recombinant bacterial strains and procedures developed at ECBC. These recombinant strains will be grown in industrial-scale fermentation systems such as the one shown below in Figure 3.

Fig. 3: Large-scale industrial fermentor

The bacterial cells will be harvested, washed, broken and the cell extracts containing the enzymes (10-50% of total protein) prepared as granules. Granules are preferable to powders because of increased stability, ease of handling, and reduction in dust levels during both production and use. An example of granules of a laundry detergent enzyme is shown in Figure 4.

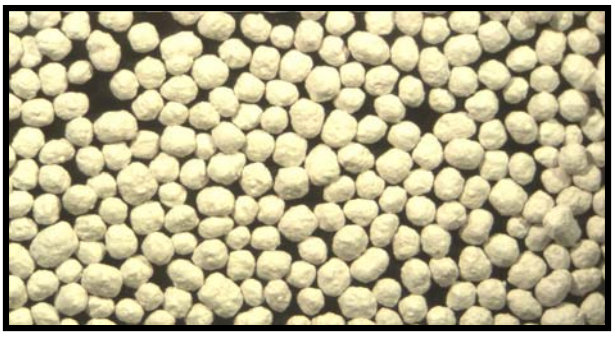

Fig. 4: Typical enzyme granules

An example of the component parts of an enzyme granule is shown in Figure 5. This particular example is of a granule that is used in laundry detergents.

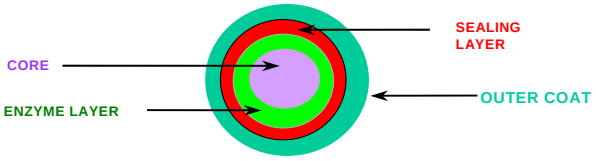

Fig. 5: Fluid bed granule example: G Granule

In this example, the inner layer is the core, which can be composed of starch, sucrose, polyvinyl alcohol (PVA) or other materials. It serves as the carrier for the enzyme and provides mechanical strength for low dust. The enzyme layer consists of the enzyme, mixed with PVA, and provides the cleaning power. In the US and Mexico, a barrier layer of ammonium sulfate is used to scavenge the chlorine from the wash water. Finally, the outer coat of TiO2, PVA and a surfactant, provides enzyme stability, low dust, and color.

The projected product specifications for the OPAA and OPH granules are:

- Particle size: 600-1000 microns
- Dissolution rate: 1 kg in 500 gallons of water in 5 minutes
- Enzyme activity: TBD (expected protein load at 6-10% per granule)
- Form: low dust, fast dissolving granule

The enzyme granules will be sold to companies that produce fire-fighting foams, sprays or other materials that can be used as decontamination vehicles. A number of these materials that are compatible with the enzymes have already been identified.

As the program progresses, collaborative work under a cooperative research and development agreement (CRADA) being negotiated with Genencor will proceed in several directions. The genes for the enzymes will be transferred into improved production host strains to increase yield and reduce cost. Eventually, other enzymes such as ones for sulfur mustard (HD), carbamate pesticides, and biological agents will be included in the scale-up program.

CONCLUSION

For many years the use of enzymes for decontamination of CBW agents was primarily considered to be a laboratory curiosity. However, with the advent of recombinant DNA technology, and significant advancements in microbiology and biochemical processing, the large-scale production and commercialization of decontaminating enzymes is now a reality.

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